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| TITLE OF THE INVENTION (500 characters max) <u>A method for the production of intervertebral disc derived chondrocyte transplants....</u> | | | | | |
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Respectfully submitted,

[Page 1 of 2]

Date February 12, 2004

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CONFIRMATION

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A method for the production of intervertebral disc derived
chondrocyte transplants and the use as a transplantation
material

Abstract

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The invention relates to a method for the in vitro
production of vital intervertebral disc chondrocyte or
cartilage transplants, and the transplantation of the in
vitro produced intervertebral disc chondrocytes and
cartilage. According to this invention, viable
chondrocytes, obtained from human and/or animal degenerated
or damaged intervertebral disc, are cultured in cell
culture vessels until a sufficient amount of cells with
original native phenotype and high proliferation and
differentiation capability are available that were then
transplanted into a degenerated or damaged disc.

Description

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The invention relates to a method for I) the in vitro
production of vital intervertebral disc derived chondrocyte
transplants, made of chondrocytes obtained from degenerated
or damaged intervertebral disc tissue of patients suffering
on spine disorders, and to do use thereof as a
transplantation material for the treatment of degenerated
or damaged intervertebral disc and for III) the in vitro

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5 production of three-dimensional, vital and mechanically
stable intervertebral disc cartilage tissue and their use
as a transplantation material for the treatment of
degenerated or damaged intervertebral disc, and for the IV)
method of transplantation, V) as well as for their use in
10 testing/screening of active substances and physical
factors.

The invention relates also to in vitro produced
intervertebral disc derived chondrocyte transplants and in
vitro produced three-dimensional intervertebral disc
15 cartilage as well as therapeutic formulations, such as
injectabilia which contain the produced tissue and cell
transplants.

The progressive intervertebral disc degeneration, caused by
20 trauma or normal aging process, leads to acute and chronic
back and leg pain as well as to instability of spine. Long-
term physical disability and a reduced quality of life are
results of progressive degeneration of disc. More than
300.000 patients in Europe suffering on spine disorders.
25 Because no effective therapies to retard or reverse disc
degeneration have yet been devised, a variety of surgical
procedures have been developed to treat disc degeneration
and back pain. Unfortunately, the procedures currently
available fail to offer an outcome that is prosthetic and
30 at the same time physiologic. They include surgical
removing of nucleus compartment with intact annulus of
discus hernia from the spinal chanel or the removing of
complete intervertebral disc following by insertion of
implants or following by fusion of both adjacent spinal
35 segments. However, the surgeries tends to limit motion by

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5 immobilisation of affected spine level and their outcome is
a lack of function at particular spine segment followed by
excessive stresses to adjacent intervertebral discs.

10 The invention describe for the first time the unique method
for the production intervertebral disc derived chondrocyte
transplants, that allows, by the transplantation of cells
into the degenerated or damaged intervertebral disc, the
regeneration of the intervertebral disc and efficient
15 maintenance of the disc matrix and therefore restore
neurological and biomechanical function disorder caused by
discus hernia of affected spine segment.

Furthermore, the invention describe for the first time the
method that allows the regeneration of disc and maintenance
of neurological, biological and mechanical function of disc
20 even in progressive intervertebral disc degeneration or
traumatological damage of outer ring of disc (annulus
fibrosus),

With the former method described is directed to the
production of intervetrebral disc derived chondrocyte
25 transplants, whereas the latter method described is focused
on the production of three-dimensional intervetrebral disc
tissue transplant, however both methods use autologous
(patient own) cells isolated from damaged intervertebral
disc.

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The autologous chondrocyte transplantation is the well
established method for the treatment of focal articular
cartilage defects by using cultured autologous articular
35 cartilage cell transplants. The method is based on

5 capability of articular chondrocyte transplants for the de
novo formation of articular cartilage in vivo.

In this method, for example, a biopsy is taken from a
patient from a region of hyaline healthy cartilage,
chondrocytes are isolated from the biopsy, propagated by
10 culturing and finally transplanted into articular focal
cartilage defect using injection. The treated cartilage
lesion site is completely filled-up with de novo synthesized
articular cartilage produced by transplanted chondrocytes.

It is well known, that cultured autologous cell transplants
15 are capable to regenerate the specific target tissue in
vivo following their in vivo application into damaged site
in the body.

The goal of Tissue Engineering in the regeneration of
intervertebral disc is the restoration of degenerated or
20 damaged disc tissue by the use of ethical and medical
innovative method: transplantation of an specific cell
transplant or transplantation of ex-vivo manufactured
three-dimensional disc cartilage. There is no published
similar methods.

25 The task of this invention was therefore to provide such a
methods for the production of intervertebral disc derived
chondrocyte transplants and methods for the production of
vital and mechanically stable intervertebral disc tissue
suitable for the transplantation and rapid disc
30 regeneration and maintenance of disc function. For the
finding/creating the described method, it was essential
that the biopsy obtaining can be performed under ethical
conditions and that the cultured disc derived cells
maintain their unchanged phenotype as well as have a high
35 proliferation rate and differentiation capacity.

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According to the described invention, the source for the manufacturing of transplant is degenerated and/or damaged intervertebral disc tissue, because no other tissue source, for example such as adjacent intact adult intervertebral disc, is available for the described treatment method, due to ethical and medical reason. Due to apoptosis of tissue-specific cells and their replacement by unspecific, various type of cells in degenerated or damaged tissue, so far it was suspected, that it is not possible to obtain sufficient number of vital cells from the degenerated or damaged tissue, which are capable to proliferate with sufficient multiplication factor and finally able to differentiate to the specific tissue and to restore intervertebral disc. Surprisingly, it has been found, that the sufficient number of vital cells can be isolated from degenerated or damaged intervertebral disc tissue and that those isolated cells also under given culturing conditions proliferate and differentiate tissue-specific and therefore are suitable for cell-based therapy for the functional restoration of damaged disc.

For the treatment of spine by the manufactured disc derived chondrocyte transplants it is important that the outer disc ring, annulus fibrosus, is already healed in that way that no liquid, such as cell suspension, can come out from the internal compartment of the disc. That time period is patient-specific. During this time period the cultured autologous disc-derived chondrocyte transplants may not change their tissue-specific phenotype regarding their

5 differentiation capability and by that the treatment success.

Furthermore, the in vitro manufactured cell- and tissue transplants should not express immunological reactions in the patient who is receiving the transplant.

10 Surprisingly, we found that this requirement can be fulfilled by using of simple method as described in claim 1.

15 According to the invention, patient-derived tissue biopsies or samples, e.g. from damaged and degenerated intervertebral discs are used as starting material. The disc-building cells are isolated from this biopsies, according to conventional methods, using enzymatic
20 digestion of the tissue, migration, or reagents recognizing the target cells. According to the invention, these cells are than subjected to culturing in suspension in a simple fashion, using conventional culture medium supplemented by patient serum, without supplementing antibiotics and
25 fungistatics as well as growth factors, in a cell culture vessels until sufficient cell number become available. The period of culturing and the number of cell passaging were reduced as much as possible in order to maintain the unchanged cell phenotype. Such phenotype changes have been
30 observed in long-term cell cultures with frequent passages (see Fig 4). After sufficient cell number has been achieved, the cell were harvested and the cell transplant as the suspension of disc-derived chondrocytes has been produced.

5 According to the invention, in an additional method, the isolated disc-derived chondrocytes have been precultured and without passaging propagated in short-term. Following this, the precultured cells have been harvested and cryopreserved until the transplantation. Before the
10 transplantation, the cell have been thawed and cultured in the conventional cell culturing medium in the presence of autologous serum until sufficient number has been achieved. Following this, the cells have been harvested and the autologous disc-derived chondrocyte transplant has been
15 produced. Surprisingly, it has been found, that the disc-derived cells did not lost through freezing, thawing and finally short-term culturing, their capability to synthesize specific matrix compounds (see Fig. 5). However, the disc-derived cells lose their capability to produce de
20 novo matrix compounds in long-term monolayer culturing up to 2-3 month without freezing.

According to the invention, in an third method, the disc biopsies have been obtained from damaged or degenerated discs of patients. The tissue-building (forming) cells are
25 isolated from the biopsies, according to conventional methods, using enzymatic digestion of the tissue, migration, or reagents recognizing the target cells. According to the invention, these cells are then subjected to stationary culturing in monolayer simple fashion, using
30 conventional culture medium, until an sufficient number is achieved. The cultured cells are then transferred in cell culture vessels with hydrophobic surface and tapering bottom and subjected to the culturing until a three-dimensional cell aggregate is formed which includes at
35 least 40% by volume, preferably at least 60% by volume and

5 up to a maximum of 95% by volume of extracellular matrix
(ECM) having differentiated cells embedded therein. The
cell aggregate having formed has an outer region wherein
cells capable of proliferation and migration are present.
10 The structure of the cell aggregates obtained according to
the invention is illustrated by the microscopic photographs
in Fig 1. Fig.1 shows a detail enlargement of the cross-
section of a according to the invention produced cell
aggregate with M as the zone of reduced cell proliferation
and the tissue-specific matrix proteins and the zone P as
15 the outer zone of proliferative and migratory cells.

It is noteworthy that all the cells derived from damaged
and degenerated disc tissue maintain their high
proliferation capacity (Fig 2.) as well as extremely high
20 differentiation potential for the synthesis of disc-
specific matrix proteins and marker proteins, such as
Aggrecan (Fig. 3a), hyalin-specific proteoglycans (Fig.
3b), collagen type I (Fig. 3c), collagen type II (Fig. 3d)
collagen type III (Fig. 3e) und protein S100 (Fig. 3f) and
25 do not lose their phenotype by freezing and subsequently
thawing (Fig. 5).

It is surprisingly, that all disc-derived cells, according
to this invention, isolated from damaged and degenerated
30 disc tissue and integrated in the three-dimensional disc-
cell aggregates produced from those isolated cells
according to the invention survive, and that the cells
integrated inside the aggregates do not necrosise even
after an advanced period of culturing. With increasing time
35 of cultivation, the cells inside the aggregates undergo

5 differentiation to form aggregates of disc cartilage tissue
consisting of ECM, differentiated disc-chondrocytes and
peripheral proliferating disc-chondrocytes. During the cell
differentiation in cell culture, the spacing of the
aggregated cells increases due to formation of the tissue
10 specific matrix. A tissue histology develops inside the in
vitro produced three-dimensional disc aggregates is highly
similar to natural tissue. During the further course of
disc aggregate production, a zone of cells capable of
proliferation and migration is formed at the boundary of
15 the disc aggregates. This zone is invaluablely advantageous
in that following incorporation of the produced disc
aggregates into the damaged intervertebral disc, the cells
located in this peripheral zone are capable of migrating to
make active contact with the surrounding tissue and/or
20 enable integration of the tissue produced in vitro in the
environment thereof. Thus, the produced disc-tissue-
specific cell aggregates are excellently suitable for use
in the treatment of damaged and degenerated intervertebral
disc and in the in vivo neogenesis of intervertebral disc.

25 Due to biomechanical loading of intervertebral discs
immediately following the treatment by disc cell
transplants and also according to the primary goal of the
treatment to restore the disc height by transplanting of
30 the disc cell transplants, it could be advantageous to use
larger, mechanically stable in vitro produced three-
dimensional tissue for the treatment. For this case, at
least two or preferably more of the cell aggregates
obtained are fused by prolonging culturing thereof under

5 the same conditions and in the same culture vessels as described above until the desired size is reached.

Fig. 4 shows five disc-cell aggregates during their fusion. 10 The boundary between single disc cell aggregates cannot be recognized any longer. Following further culturing, the disc cell aggregates are completely fused and a larger disc tissue patch has formed. The structure of the larger disc cell aggregates thus obtained is identical to that of the 15 single disc cell aggregates obtained initially. They may include ECM up to a maximum of 95%, and all of the cells included in the piece of tissue obtained exhibit vitality.

A medium usual both for suspension and monolayer culture, 20 e.g. Dulbecco's MEM supplemented with serum, can be used as a cell culture medium. It is preferred to use DMEM and HAMS at a ratio of 1:1. However, to avoid an immunological response of the patient to the tissue produced in vitro, it is preferred to use autogenous/autologous serum from the 25 patient as serum. It is also possible to use xenogeneic or allogeneic serum.

According to the invention, no antibiotic, fungistatic 30 agents or other auxiliary substances are added to the cell culture medium. It has been found that only autogenous, xenogenic or allogenic cultivation of the cells and cell aggregates and cultivation in absence of antibiotic and fungistatic agents allow for non-affected morphology and 35 differentiation of the cells in the monolayer culture and undisturbed formation of specific matrix within the cell

5 aggregates. Furthermore, by avoiding any additive during the production, any immunological reaction is excluded following incorporating the in vitro produced tissue in a human or animal organism.

10 The size of in vitro produced disc cartilage depends on the number of introduced cells per volume of culture medium. For example, at incorporating 1×10^7 cells in 300 μ l culture medium, three-dimensional disc cartilage aggregates with approximately 500-700 μ m diameter, are formed within one
15 week. Another way would be in vitro fusion of small cell aggregates to form larger ones - as described above - and incorporation of the latter in the defect. According to the invention, it is preferred to use between 1×10^4 and 1×10^7 cells in 300 μ l culture medium to produce the small
20 cell aggregates, more preferably 1×10^5 cells. Depending on the cell type and patient-specific characteristics, the cell aggregates having formed after several days are then subjected to the culturing for at least 2-4 weeks to induce formation of the tissue specific matrix. From about one
25 week of culturing on, it is possible to fuse individual disc cartilage tissue in special cases, so as to increase the size of tissue patch.

30 As cell culture vessels, the inventive cultivation in suspension requires the use of those having a hydrophobic, i.e., adhesion-preventing surface, such as polystyrene or Teflon. Cell culture vessels with a non-hydrophobic surface can be made hydrophobic by coating with agar or agarose. Further additives are not required. Preferably, well plates
35 are used as cell culture vessels. For example, 96-well

5 plates can be used to produce small cell aggregates, and
24-well plates to produce fused larger aggregates.

10 The invention is also directed to therapeutic formulations
comprising the intervertebral disc chondrocyte transplants
and intervertebral disc cartilage transplants according to
the invention, e.g. injection solutions.

15 The invention is also directed to the use of intervertebral
disc chondrocyte transplants and intervertebral disc
cartilage transplants of the invention for the
testing/screening various factors, e.g. active substances
20 and physical factors having an effect (influencing) on the
formation of matrix and differentiation of cells. For this
purpose, the intervertebral disc chondrocyte aggregates are
produced according to the invention, the potential drugs,
therapeutic substances and compounds and medicinal products
25 to be tested are added at various stage of maturity, and
most various parameters of in vitro intervertebral disc
tissue formation and maturation are characterized. Compared
to conventional drug testing using animals or tumour
systems, these tests are highly patients-specific and
30 enable individual results and diagnosis as a result of
using autologous material only.

Without intending to be limiting, the invention will be
illustrated in more detail below with reference to the
examples.

Examples

Example 1: In vitro production of intervertebral disc chondrocyte transplants

A biopsy is taken from damaged or degenerated intervertebral disc tissue. Disc-derived chondrocytes are isolated from this biopsy using enzymatic digestion by incubation with collagenase solution. Following separation of the isolated cells from the undigested tissue, the cells are transferred in cell culture flasks and, following addition of DMEM/HAMS F12 culture medium (1:1) and 10% autologous serum from the patient, incubated at 37°C and 5% CO₂. The medium is exchanged twice a week. After reaching the confluence stage, the cell layer is washed with physiological saline solution and harvested from the cell culture surface using trypsin. Following subsequently washing, the disc-derived chondrocytes are transferred into physiological saline solution and the disc chondrocyte transplant is so available for the transplantation.

The differentiation capacity of disc-derived chondrocytes in the released disc chondrocyte transplant has been shown in an in vitro model. Due to the disc-specific matrix proteins and marker proteins, expressed in the cells of the released disc chondrocyte transplants, the disc-specific tissue structure is formed (Fig.3)

Example 2: Transplantation of intervertebral disc chondrocytes

The produced intervertebral disc chondrocyte transplants, as described in example 1, containing minimally 1.000, max. 100 Million cells, preferably approx. 1 Million

5 . intervertebral disc chondrocytes, were taken up in physiological saline solution and injected into a interspace of damaged and/or degenerated intervertebral disc. It has been shown/determined that in the treated intervertebral disc, the water content increases and the
10 disc height can be maintained, due to the transplanted intervertebral disc chondrocytes producing de novo matrix proteins. According to the invention, the in vitro produced disc chondrocyte transplants are well accepted from the from the treated patient. The in vitro produced disc
15 chondrocyte transplants ensures rapid integration of the proliferating and migrating disc cells as well as the regeneration of disc tissue through their differentiation capability.

Therefore, the disc chondrocyte transplants allow with
20 their features the rapid regeneration of the intervertebral disc, the rapid healing/recovery of the patient and the rapid functional restoration of the intervertebral disc.

25 Example 3: in vitro production of intervertebral disc tissue

A biopsy is taken from damaged or degenerated intervertebral disc tissue. Disc-derived chondrocytes are isolated from this biopsy using enzymatic digestion by
30 incubation with collagenase solution. Following separation of the isolated cells from the undigested tissue, the cells are transferred in cell culture flasks and, following addition of DMEM/HAMS F12 culture medium (1:1) and 10% autologous serum from the patient, incubated at 37°C and 5%
35 CO₂. The medium is exchanged twice a week. After reaching the confluence stage, the cell layer is washed with

5 physiological saline solution and harvested from the cell culture surface using trypsin. Following subsequently washing, 1×10^5 disc-derived chondrocytes each time are transferred into cell culturing vessel coated by agarose. After one day, the first cells arrange into aggregates. 10 Those aggregates are supplied with fresh medium every second day and cultured for at least 2 weeks.

In those, according to the invention, in vitro produced intervertebral disc tissue, the expression and deposition of disc-specific matrix compounds and marker proteins such as Aggrecan (Fig. 3a), hyalin-specific proteoglykanes (Fig. 15 3b), Collagen Typ I (Fig. 3c), Collagen Typ II (Fig. 3d), Collagen Typ III (Fig. 3e) und Protein S100 (Fig. 3f) is shown. Those molecules are components of the native disc tissue in vivo, representing the most important structural 20 proteins which are of crucial significance for disc function.

Example 4: Transplantation of produced disc tissue

25 The disc tissue produced as described in example 3 (approx. 10 to 1000 patches), preferably up to 100 patches were taken up in physiological saline solution and injected into the intervertebral space of progressively degenerated and/or strong traumatically damaged intervertebral disc with destroyed annulus fibrosus. It has been determined, 30 that, in according to the invention, in vitro produced intervertebral disc tissue is well accepted from the treated patient and that the transplanted tissue allows, beside the mechanical restoration of the affected disc 35 tissue, also rapid integration into that tissue due to

5 proliferating and migrating cells in outer periphery of
aggregates, as well that transplanted tissue allows the
regeneration of affected tissue due to differentiation
capacity of the cells in the transplant. Therefore, the
structure and the function of the in vitro produced disc
10 tissue allow rapid regeneration of the affected disc
tissue, the rapid healing and recovery of the patient and
the rapid restoration of the disc function.

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Claims

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1. A method for the production of disc chondrocyte transplants, characterized in that disc chondrocytes are isolated/obtained from a damaged, degenerated and sequestered intervertebral disc tissue, and these cells are grown under maintaining their phenotypical characteristics, that those cells can differentiate and therefore are able to regenerate the intervertebral disc tissue following their transplantation.

2. The method according to claim 1, Characterized in that the intervertebral disc chondrocytes can be isolated from a damaged, degenerated and sequestered intervertebral disc tissue

3. The method according to claim 1, Characterized in that the isolated intervertebral disc chondrocytes are grown under strictly autologous culture conditions, e.g. only supplementing autogeneous, patient-own, serum

4. The method according to claim 1, characterized in that the isolated intervertebral disc chondrocytes are grown in that way, that their phenotypical features regarding their capacity to synthesize the matrix proteins and marker proteins remain unchanged.

5 5. The method according to claim 1,
characterized in that,
the isolated intervertebral disc chondrocytes after
initial short-term culturing can be frozen and
subsequently thawed, without changing their
10 phenotypical features regarding their capacity for
synthesis of matrix-specific proteins and marker
proteins.

15 6. The method according to claim 1
Characterized in that,
the isolated intervertebral disc chondrocytes, grown in
a monolayer culture, express their capability for the
formation of extracellular matrix comprising of disc-
matrix-specific proteins.

20 7. The method according to claim 1,
characterized in that,
the isolated intervertebral disc chondrocytes, initially
grown in a monolayer culture, and subsequently frozen and
25 thawed, maintain their capacity for the formation of
extracellular matrix comprising of disc-matrix-specific
proteins.

30 8. The use of intervertebral disc chondrocyte transplants
according to claim 1 as autogenous, xenogeneic or
allogeneic transplantation material for the treatment
of degenerated intervertebral disc.

- 5 9. A therapeutic formulation, comprising
 intervertebral disc chondrocyte transplants according
 to claim 1.
- 10 10. The method according to claim 1,
 characterized in that,
 the intervertebral disc chondrocyte and in vitro
 produced intervertebral disc tissue also can be
 cultured with addition of growth-stimulating compounds.

Abstract

The invention relates to a method for the in vitro production of intervertebral disc chondrocyte transplants, produced from disc chondrocytes isolated from damaged, degenerated and sequestered intervertebral disc, where the cells can be grown without changes of their specific features, and the cells maintain their proliferation and differentiation capacity regarding the formation of intervertebral disc-specific matrix and the cells can be transplanted.

The invention is also directed to the in vitro produced intervertebral disc tissue and therapeutic formulations, e.g. injection solutions comprising such tissue.

Surgical procedure for the autologous disc-derived chondrocyte transplantation (ADCT)

This surgical intervention is the application of manufactured disc-derived chondrocyte transplants - transplantation- into the damaged disc. It has to be performed after healing and achieving of stability of the ligamentum longitudinale posterior.

Surgical procedure has to be performed in surgical sterile tract marking of the intervertebral disc space on the patient back under fluoroscopy control into 2 dimensions,

5 following skin disinfection, covering the skin areal with a sterile foil under use of superficial local anesthesia with Xylocain. 1% or comparable drug.

10 It follows determination of the syringe volume for rinsing after the cell instillation, puncture of the opposite side of the former surgical approach, insertion of the Initial-Puncture-Needle-Threesquare with lancet,, removing of the lancet, positioning of the penetration needle, manipulations under fluoroscopy control, performance of the pressure measurement according to the pressure measurement
15 working guideline, resuspension of the cryo-vial with the cell suspension, instillation of the cells without using contrast media and without (not recommended but possible) radiological control to avoid adverse effects on the cells, injection of 0,1 ml NaCl-solution to rinse remained cells
20 out of the syringe, removing of the application system and local wound care.

Post operative care

25 Includes up to 3 days hospitalization with up to 12 hours strict bed rest, following 12 to 24 hours regular bed rest, following 24 to 48 hours bed rest with isometric exercises (physiotherapy, site positioning, abdomen positioning possible). From 2nd to 21st day a stable lumbar orthosis is
30 prescribed.

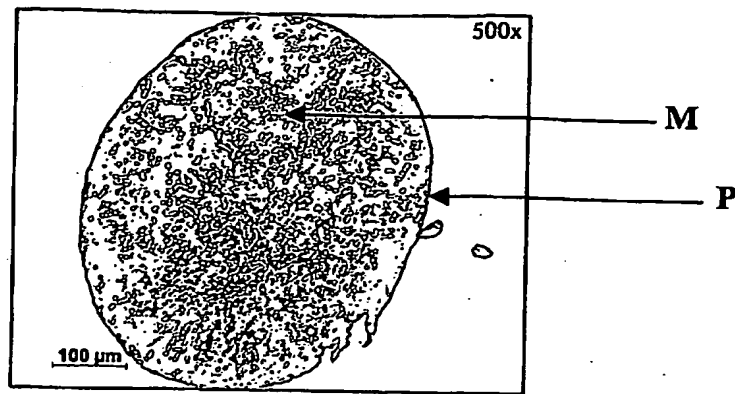


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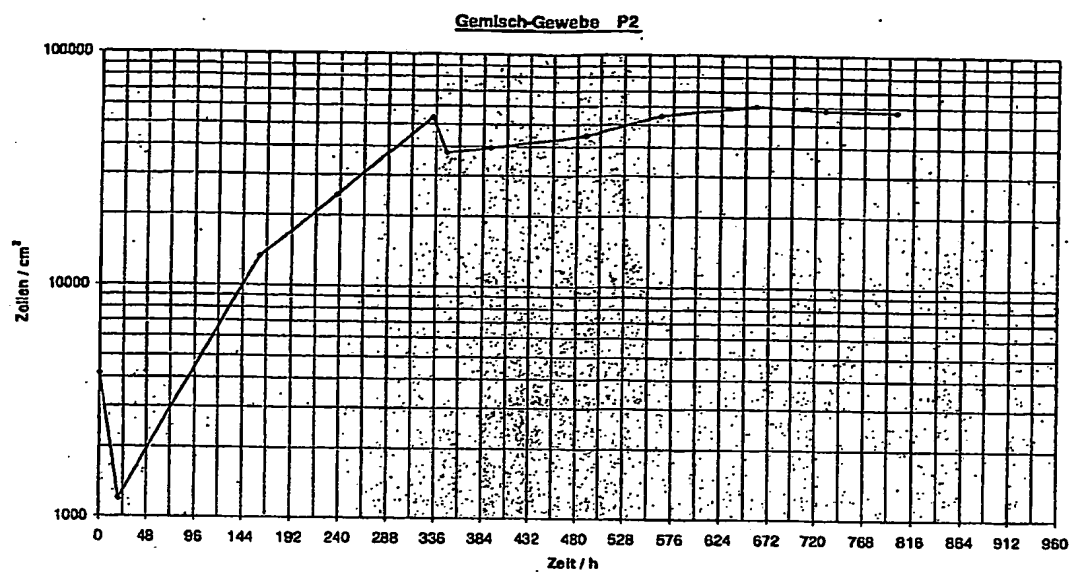


Abb.2

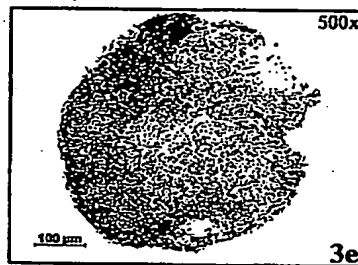
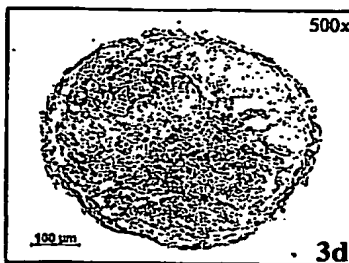
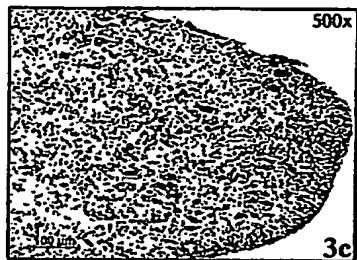
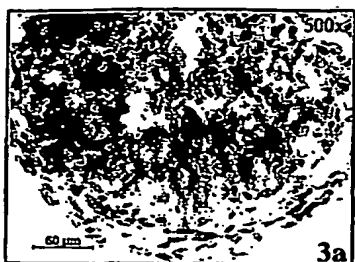


Abb.3

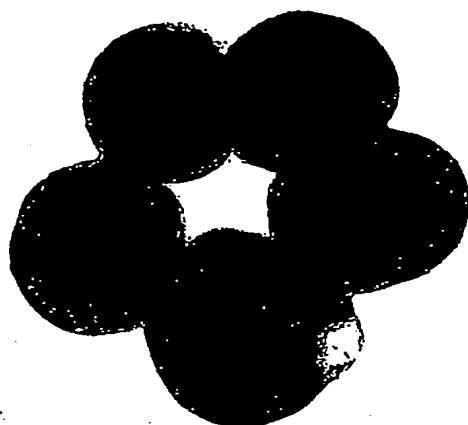


Abb.4

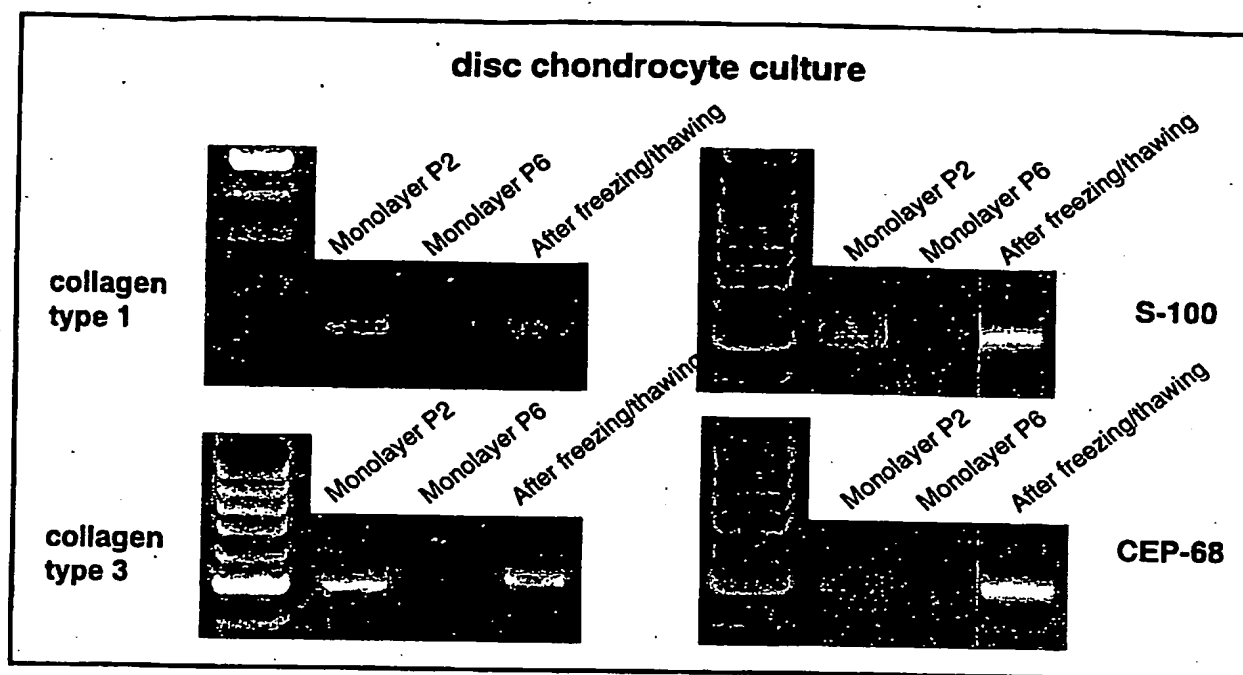


Fig. 5: Expression of different matrix and regulative proteins by disc derived chondrocytes cultured in monolayer for different passages and cultured in monolayer after freezing and thawing of cells. Monolayer passage 2 (P2), Monolayer passage 6 (P6), after freezing and thawing (after freezing/thawing).